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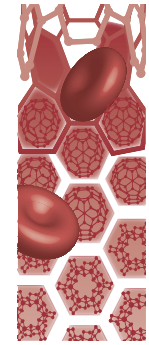

Nanoformulations of a potent copper-based aquaporin inhibitor with cytotoxic effect against cancer cells

Aim: Development of liposomal formulations of Cuphen, a potent copper-based aquaporin inhibitor with therapeutic potential against melanoma and colon cancer. **Materials & methods:** Cuphen was incorporated into liposomes using the dehydration–rehydration method. The ability of Cuphen to induce cancer cell death was evaluated by MTS and ViaCount assays. *In vivo* toxicity studies were performed in BALB/c mice. **Results:** *In vitro* studies illustrated the antiproliferative effects of Cuphen in different cancer cell lines, in free form or after incorporation into liposomes. *In vivo* studies revealed no toxic effects after parenteral administration of Cuphen liposomes. **Conclusions:** Cuphen liposomes are highly attractive to be further tested in murine models due to the possibility of stabilizing and specifically deliver this metallodrug to tumor sites.

First submitted: 2 March 2016; Accepted for publication: 19 May 2016; Published online: 7 July 2016

Keywords: aquaporins • colon cancer • Cuphen • cytotoxic effect • *in vivo* toxicity studies • liposomes • melanoma • metallodrugs

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Introduction

Aquaporins (AQPs) are a family of small transmembrane proteins involved in the transport of water (orthodox AQPs) and small solutes such as glycerol (aquaglyceroporins), across the cell plasma membrane [1,2]. In mammals, 13 different AQP isoforms (AQP0–12) can be found widely distributed throughout the body, playing several roles in different physiological processes, such as urinary concentrating mechanism, glandular secretion or skin hydration. Some studies have demonstrated altered human AQP distribution and expression in pathological conditions and, thus, their modulation can be useful in treating human diseases. In the last decade, an increasing number of reports showed that AQPs are abundantly expressed in different tumors and could serve as biomarkers with prognostic value of cancer aggressiveness [3–5].

AQPs are crucial for tissue water balance in response to osmotic gradients, essential to maintain cell function, including in malignant cells. By facilitating water fluxes driven by an increase in local osmolarity due to transmembrane ion fluxes at the leading edge of the migrating cell, AQPs may facilitate tumor growth, local infiltration and metastasis. AQPs have also been associated with tumor proliferation through their ability to enable glycerol uptake that favors cellular energetic metabolism, biosynthesis and, consequently cell division [4,5]. Therefore, AQP expression can be advantageous for high metabolic turnover or tumor-specific metabolic pathways needed for survival of malignant cells.

As an example, the aquaglyceroporin AQP3 that is normally expressed in the skin with a role in skin hydration [6], was found to be overexpressed in cancerous human prostate cells [7], primary squamous cell carcinomas [8] and in carcinogenesis and progression of several different carcinomas. In particular, Hara-Chikuma and Verkman have dem-

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onstrated an aberrant AQP3 expression in skin carcinomas, while AQP3-null mice showed tumorigenesis resistance [9]. Although the mechanisms by which AQP3 interferes with cell differentiation and participates in tumorigenesis are not completely clear, they rely on AQP3 glycerol transport allowing increased ATP synthesis and promoting cell proliferation and tumor spread [9,10].

Due to the involvement of AQPs in cell migration, proliferation and adhesion in human cancer, these proteins emerge as promising drug targets and their modulators as useful antitumor agents [11,12]. While there is still much to learn on the pathological processes arising from AQPs mutations or dysfunction, pharmacotherapy via AQPs modulation was proposed as a valuable therapeutic strategy.

At present, there are few selective AQPs inhibitors described [11]. Moreover, the majority fails to undergo to clinical trials due to extreme toxicity. Recently, Martins *et al.* described new gold-based coordination compounds as potent and selective inhibitors of the glycerol transport through AQP3 in human red blood cells (hRBC) [13,14]. Interestingly, all these gold complexes contain N-donor ligands and are also described as antiproliferative agents against cancer cells *in vitro* [15–17]. Notably, no cytotoxic effect of the lead compound [Au(phen)Cl_2] (phen=1,10-phenanthroline, Auphen, Figure 1) was observed for tumor cells that do not express AQP3 [18]. Furthermore, other gold complexes were also tested as potent inhibitors of cancer cells growth and they appeared as promising drug candidates [15,19].

Driven by these recent results regarding AQP3 inhibition by gold compounds, in the present work a small library of potent and selective AQP3 metal-based inhibitors – including both gold(III) and copper(II) complexes (Figure 1) – were first tested against a human skin carcinoma cell line. Out of the obtained results, the copper-based complex [Cu(phen)Cl_2] (Cuphen) was selected for subsequent *in vitro* experiments. Hence, different human and murine cell lines were considered, aiming at establishing murine models of melanoma and/or colon cancer.

It is worth mentioning that several copper-based compounds have already been reported for their anticancer properties. In this context, the results by Ruiz *et al.* hold great promise, and include the group of metal compounds named *casiopeinas*, copper-based coordination complexes – containing a phenanthroline-type ligand and with generic structure of $[Cu(N–N)(O–N)]^+$ or $[Cu(N–N)(O–O)]^+$ – that have proven to be cytotoxic to cancer cells, sensitive or resistant to cisplatin, to xenograph tumors in mice, and are now in clinical trials [20–23]. For these copper-based drugs, still the mechanism of action is not fully elucidated; most importantly, a number of side effects have been reported, including hematotoxicity [24]. In fact, Cu(II) ions bound to DNA react with H_2O_2 and ascorbic acid generating hydroxyl radicals, which immediately attack DNA bases in a site-specific manner, and thus the availability of Cu(II) ion *in vivo* may also be potentially harmful.

The development of metallodrugs in the treatment of cancer has been hampered by the low efficiency of transport processes of their active species to biological targets. This has led to the design and development of novel technologies based on nanostructured materials, acting mainly as vectors for metallodrug delivery or simply as protectors of active species of the complexes for amplifying their activities and reducing their degradation [25,26]. In the case of anticancer copper complexes, few reports in the literature describe their delivery via TiO_2 nanoparticles or via conjugation to phosphorus dendrimers [27].

To the best of our knowledge, no use of liposome formulations has been attempted so far for cytotoxic copper(II) complexes. Liposomes are widely recognized the drug delivery system that may allow the minimization of drawbacks commonly presented by various anticancer agents, including metallodrugs, such as drug instability and reactivity [28]. They are also able to protect the incorporated drugs from premature degradation/metabolization, while keeping their therapeutic activity [25,29–31]. In addition, liposomes mimic cellular membranes conferring biocompatibility and

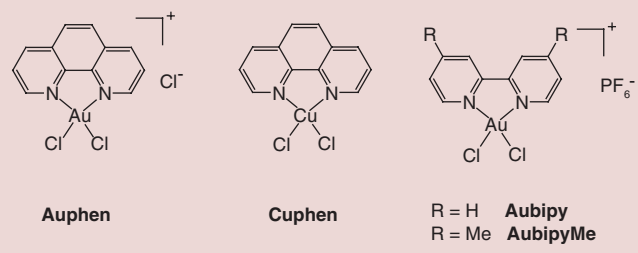

Figure 1. Chemical structures of the investigated gold(III) and copper(II) complexes.

biodegradability and may allow their accumulation at tumor sites characterized by impaired lymphatic function [32,33].

Taking into account that Cuphen presents a copper center with possible toxic effects, and in the process of stabilizing the metal compound for *in vivo* administration, the compound was first incorporated in different liposomes and the antiproliferative activity induced by the most promising formulations was evaluated against the same cancer cell lines *in vitro*. In addition, the blood toxicity of Cuphen and of its liposome formulations was assessed, and preliminary *in vivo* studies were conducted to study the safety of liposomal formulations for further therapeutic evaluation in cancer murine models.

Materials & methods

Chemical products

The metal complexes included in the present work were [Au(phen)Cl_2] (phen=1,10-phenanthroline, Auphen), [Cu(phen)Cl_2] (Cuphen) and two different compounds of general structure [Au(bipy)Cl_2]PF_6 (bipy = 2,2'-bipyridine, Aubipy, or 4,4'-dimethyl-2,2'-bipyridine, AubipyMe) (Figure 1). Cuphen is commercially available (Sigma-Aldrich), while the gold(III) complexes were prepared according to literature procedures [34,35]. The purity of the compounds was confirmed by NMR and elemental analysis, and all of them showed purity greater than 98%.

The pure phospholipids, egg phosphatidylcholine (ePC), dimyristoyl phosphatidylcholine (DMPC), dipalmitoyl phosphatidylcholine (DPPC) and distearoyl phosphatidylethanolamine covalently linked to poly(ethylene glycol) 2000 (PEG), used for the preparation of liposomal formulations were purchased from Avanti Polar Lipids (AL, USA). Deionized water (Milli-Q system; Millipore, Tokio) was used in all experiments. Nuclepore Track-Etch Membranes were purchased from Whatman Ltd (NY, USA). Culture media and antibiotics were obtained from Invitrogen (Life Technologies Corporation, NY, USA). Reagents for cell proliferation assays were purchased from Promega (WI, USA). Octadecylamine (*n*-Stearylamine [SA]), cholesterol (Chol) and Hoescht 33258 were purchased from Sigma (Sigma-Aldrich, MO, USA). All the remaining chemicals and substrates used were of analytical grade.

Animals

Male BALB/c mice were obtained from Gulbenkian Institute of Science, Portugal. Animals were kept under standard hygiene conditions, fed commercial chow and given acidified drinking water ad libitum. All animal experiments were carried out with the permission of the local animal ethical committee of the Faculdade de Farmácia, Universidade de Lisboa in accordance with the EU Directive (2010/63/UE) and Portuguese laws (DR 113/2013).

Cell culture

Human epidermoid carcinoma (A431), human melanotic neuroectodermal tumor (MNT-1), human keratinocytes (HaCaT) and murine melanoma (B16F10) cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with high-glucose (4500 mg/l), supplemented with 10% fetal bovine serum and 100 µg/ml penicillin/streptomycin (Invitrogen). Murine colon cancer cells (C26) were maintained in RPMI 1640 with the supplementation described above. All cell lines were kept at 37°C under a 5% CO_2 atmosphere. Maintenance of cultures was performed every 2/3 days, until cells reached a confluence of about 80%. Cells were seeded in 96-well plates at 7.5×10^3 cells/well for MTS assays and in 24-well plates at 7.5×10^4 cells/well for Guava ViaCount assays.

Cuphen quantification in liposomes

Cuphen was quantified by UV–Vis spectrophotometry at 272 nm. Linearity of calibration curves was ensured from 2 up to 20 µM (R^2 = 0.9983; Slope: 0.0331 ± 0.0017; Y-intercept [x = 0]: 0.0001 ± 0.0020). The Cuphen liposomes quantification was preceded by liposome disruption with absolute ethanol.

Liposomes preparation

Liposomes composed of the selected phospholipids were prepared by the dehydration–rehydration method [36] (Supplementary Figure S1). Three neutral phospholipids – ePC, DMPC and DPPC were used as major constituents of Cuphen liposomal formulations. In order to play with membrane fluidity, Chol was included in the lipid composition. The addition of positively charged surfactant, SA, was also used and the influence of its presence in Cuphen incorporation parameters was compared with neutral nanoformulations. In addition, as it is our intention to develop long circulating liposomes, another Cuphen liposomal formulation containing in the lipid composition the polymer PEG covalently linked to distearoylphosphatidyl ethanolamine was also prepared. All the nanoformulations were prepared at an initial lipid concentration of 20 µmol/ml.

Briefly, the selected phospholipids were dissolved in chloroform and the lipid solution was evaporated (Buchi R-200 rotary evaporator, Switzerland) to obtain a thin lipid film in a round-bottom flask. The lipid film was then dispersed in a Cuphen aqueous solution (500 µM) and the so-formed suspension

was frozen (-70°C) and lyophilized (Freeze dryer, CO, USA) overnight.

The rehydration of the lyophilized powder was performed with a buffer constituted of 10 mM HEPES and 145 mM NaCl, pH 7.4 (HEPES buffer) in two steps, in order to enhance the Cuphen incorporation [37]. The so-formed liposomal suspension was then filtered, under nitrogen pressure (10–500 lb/in^2), through polycarbonate membranes of appropriate pore size (0.8, 0.6, 0.4 [3×], 0.2 [3×] and 0.1 [3×] µm) until an average vesicle size of 0.1 µm was obtained using an extruder device (Lipex: Biomembranes Inc., Vancouver, Canada). The separation of nonincorporated Cuphen was performed by ultracentrifugation at 250,000×*g* for 120 min at 15°C in a Beckman LM-80 ultracentrifuge (Beckman Instruments, Inc, CA, USA). Finally, the pellet was suspended in HEPES buffer, according to the final concentration desired. Empty liposomes were also prepared with the same lipid compositions. After rotary evaporation, the lipid film was dispersed in HEPES buffer and submitted to the extrusion steps as previously referred.

Liposome characterization

Liposomes were characterized in terms of lipid composition and by the following incorporation parameters: initial and final Cuphen to lipid ratios ((Cuphen/Lip)i and (Cuphen/Lip)f, respectively) and incorporation efficiency (I.E.) defined as the percentage of [(Cuphen/Lip)f]/[(Cuphen/Lip)i]. Cuphen was quantified spectrophotometrically at 272 nm (e = 33,000 M^{-1} cm^{-1}) after disruption of the liposomes with ethanol. Lipid content was determined using a colorimetric technique described by Rouser *et al.* [38]. Briefly, samples (in triplicate) containing phosphate quantities between 20 and 80 nmol (sample volume below 100 µl) were pipetted into 15-ml glass tubes. In parallel, a calibration curve with phosphate amounts ranging from 20 to 80 nmol was also pipetted in triplicate into glass tubes. All tubes were heated (180°C) in a heating block until dryness. After cooling, 0.3 ml of perchloric acid (70–72%) was added to all tubes. In order to avoid volume losses, marbles were placed on the top of all glass tubes. At this point, all tubes were heated at 180°C for 45 min to convert all the organic lipid phosphate to the inorganic form. After cooling samples to room temperature, 1.0 ml of H_2O, 0.4 ml of hexa-ammonium heptamolybdate solution [1.25% (w/v)] followed by 0.4 ml of ascorbic acid solution [5% (w/v)] were added to all glass tubes. A blue color solution was obtained due to the reduction of ascorbic acid during heating in a boiling water bath for 5 min. After cooling, the absorbance of all samples was recorded at 797 nm) in a UV-mini 1240 spectrophotometer (Shimadzu). The amount of phosphate in samples was obtained through the calibration curve with the aid of linear regression. The calibration curve was linear up to absorbance values of 1.0.

Liposomes mean size was determined by dynamic light scattering (Zetasizer Nano S [Zen 1600], Malvern Instruments, UK) at a standard laser wavelength of 663 nm. The system also reports a polydispersity index, as a measure of particle size distribution, ranging from 0.0 for an entirely monodisperse sample up to 1.0 for a polydisperse suspension (Supplementary Figure S2). Zeta potential of liposomal formulations was measured in a hydrodynamic sizing system (Zetasizer Nano Z [Zen 2600], Malvern Instruments).

Cell viability & cytotoxicity

Cell viability was evaluated in the absence (control) or the presence of increasing concentrations of Cuphen in free and liposomal forms studied by measuring mitochondrial activity, based on the colorimetric method of reducing the compound MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium), included in the commercial kit, from Promega Corporation (WI, USA). This method consists on the conversion of MTS salt and its reduction, within the mitochondria, to formazan crystals that are soluble in tissue culture. This compound can be spectrophotometrically quantified and the respective absorbance values at 490 nm are proportional to mitochondrial activity, and, consequently, to the number of living cells [39].

For the determination of cytotoxic effect of Cuphen in free and liposomal forms, cells at a concentration of 7.5 × 10^3 cells/ml were placed in 96-well plates (200 µl) for 24 h in culture conditions specified above. Afterward, culture medium was removed and adherent cells were treated with metallodrugs in free or in liposomal forms, at concentrations ranging from 0.25 to 60 µM for the initial cytotoxicity screening and 0.625 to 20 µM for the subsequent experiments. Negative control was the cell line in the presence of culture medium. Unloaded liposomes constituted another control group, using the same lipid concentrations as in Cuphen liposomes. The incubation periods were 48 and 72 h. After this period, the culture medium was removed from all wells and replaced with 100 µl of incomplete culture medium. Subsequently, 20 µl of MTS was added to all wells, agitated, followed by an incubation period of 60 min, under the same culture conditions mentioned above. Absorbance was measured at 490 nm in a microplate reader Model 680 (Bio-Rad, CA, USA). All measurements were performed with six samples for each concentration tested.

Guava ViaCount assay

ViaCount Assay distinguishes between viable and nonviable cells based on different permeability of two DNA-binding dyes in the Guava ViaCount® Reagent (Merck Millipore, Darmstadt, Germany). The nuclear dye stains only nucleated cells, while the viability dye brightly stains dying cells. MNT-1 cells were seeded in 24-well plates (7.5×10^4 cells/well) and after 24 h they were incubated with Cuphen in free and liposomal forms for 72 h. After treatment, cell culture supernatants were collected and adherent cells were detached with TrypLE (Invitrogen). Subsequently, detached cells were added to the respective supernatant and centrifuged for 5 min at 650×*g*. Supernatants were discarded and cells were suspended in 30 µl of phosphate buffered saline (PBS) with 2% fetal bovine serum (FBS). On a 96-well plate, 15 µl of cell suspension was then incubated with 135 µl of Guava ViaCount reagent for 5 min at room temperature. Data acquisition and analysis were performed using the ViaCount software module on a Guava EasyCyte 5HT Flow cytometer (Guava Technologies, Inc, CA, USA) [40].

Hemolytic activity

The hemolytic activity of Cuphen in free and liposomal forms as well as in unloaded liposomes was determined using EDTA-preserved peripheral human blood [41]. Human peripheral blood, EDTA-preserved, was obtained from voluntary donors and used in the same day of experiments. Serum was removed by centrifugation at 1000×*g* for 10 min and the erythrocyte suspension was washed three-times in PBS at 1000×*g* for 10 min. All formulations under study were diluted in HEPES buffer. Cuphen concentrations ranging from 0.2 to 200 µM were distributed in 96-well plates (100 µl/well). Then, 100 µl of erythrocyte suspension was added to all samples, microplates were incubated at 37°C for 1 h and then centrifuged at 800×*g* for 10 min. Absorbance of supernatants was measured at 550 nm with a reference filter at 620 nm. The percentage of the hemolytic activity for each sample was calculated comparing each individual determination to a 100% hemolysis (erythrocytes in distilled water), positive control and negative control (erythrocytes in PBS) according to the mathematical formula:

(AbsS – AbsN)/(AbsN – AbsP) × 100, where AbsS is the Average absorbance of the sample, AbsN is the Average absorbance of the negative control and AbsP is the Average absorbance of the positive control.

In vivo toxicity profile in BALB/c mice

The toxicity of Cuphen in free and liposomal forms was evaluated in male BALB/c mice with the age of 6–8 weeks. Groups of five mice each were injected intravenously (iv.) with formulations at a dose of 1.5 mg/kg of body weight, three-times a week during 1 week. The activity levels of animals were monitored in the analyzed period. Two days after the last injection, mice were sacrificed and blood and organs were collected and analyzed. The tissue index was calculated according to the formula:

$$\text{Tissue index} = \sqrt{\frac{\text{organ weight}}{\text{animal weight}}} \times 100$$

Statistical analysis

All data presented are expressed as mean ± standard deviation (SD) with the exception of the cytotoxicity results where data are presented as the best fit value ± standard error (SE) of at least three different experiments. Statistical analysis was performed with the paired Student's t-test or one-way analysis of variance (ANOVA) followed by Turkey's test using GraphPad Prism version 5.00 for Windows, GraphPad Software, CA, USA (www.graphpad.com). $p < 0.05$ was considered statistically significant.

Results & discussion

Antiproliferative effects of metal compounds in cancer cells

In a previous work, the antiproliferative effect of the gold(III) complex Auphen, with human AQP3 inhibition properties, was demonstrated against a human skin carcinoma cell line A432 [18]. In an attempt to select other potential cytotoxic metallodrugs, also acting as AQP3 inhibitors, an *in vitro* screening was performed where the same epidermoid carcinoma A431 cells were incubated for 72 h with Auphen, Cuphen, AubipyMe and Aubipy, with concentrations ranging from 0.250 up to 60 µM, and cell viability determined

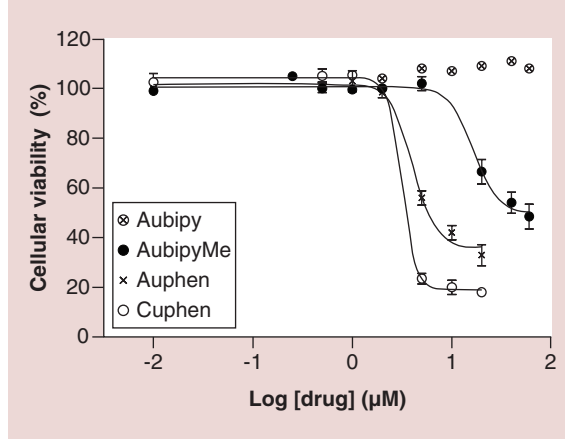

Figure 2. Concentration-dependent inhibition of A431 cellular viability 72 h after incubation with different metallodrugs (MTS assay). Results are expressed as the mean percentage (%) of control ± SD.

Table 1. Half-inhibitory concentration of metal compounds in cellular proliferation of A431 cells after 72 h incubation and inhibition of glycerol permeability in human red blood cells by the same metal complexes.

Compound	Inhibition of cell viability	Inhibition of glycerol permeation in hRBC*
	IC_{50} ± SE (μM)	
Auphen	3.7 ± 1.2	0.8 ± 0.1
Cuphen	3.0 ± 0.4	81.9 ± 4.1
Aubipy	>60	2.3 ± 0.7
AubipyMe	14.9 ± 1.1	1.0 ± 0.4

hRBC: Human red blood cell; IC_{50}: Half-inhibitory concentration; SE: Standard error.
*Data taken from [13,14].

by the MTS assay as described in the Experimental section. Dose-response curves are shown in Figure 2 and the IC_{50} values obtained from data fits are summarized in Table 1. Inhibition of glycerol permeability in hRBC by the same metal complexes [13,14] is also reported for comparison purposes.

As shown in Figure 2, the cellular behavior under the influence of the phenanthroline compounds (Auphen and Cuphen) differs significantly from the bipyridine cores ($p < 0.001$). For the compound AubipyMe, only moderate cytotoxic effects were observed. Instead, the phenanthroline derivates, Auphen and Cuphen, display similar and significant cytotoxic effects with IC_{50} values in the low micromolar range. It is worth observing that part of its cytotoxic effect may be due to the intrinsic toxicity of this phenanthroline ligand, as already described in the literature [42]. In addition, Cuphen induces a decrease in cellular viability of approximately 80%, while Auphen is only capable of a 65% reduction. Aubipy was not able to exert any toxic effect on the cellular viability even at the highest concentration of 60 μM. These differences on the cytotoxic profiles rely on the metal core, as well as on the differences in its stability with respect to both redox and ligand exchange reactions, also due to the ligands. Taking into account the results above, the cytotoxic potential of the studied metallodrugs, regarding this A431 cell line, can be represented in the following order: Cuphen>Auphen>AubipyMe.

In terms of possible mechanisms of action, it is important to highlight that copper–phenanthroline compounds are already described as potent inducers of oxidative DNA damage [43–45], and Cu^{2+} compounds, including Cuphen, are also reported as AQP3 inhibitors (Table 1) [14,46].

Taking into account these cell viability results, and due to its promising AQP3 inhibition properties, Cuphen was selected to be tested in the following studies. Cuphen cytotoxic activity was evaluated against different mammalian cell lines, all representative of

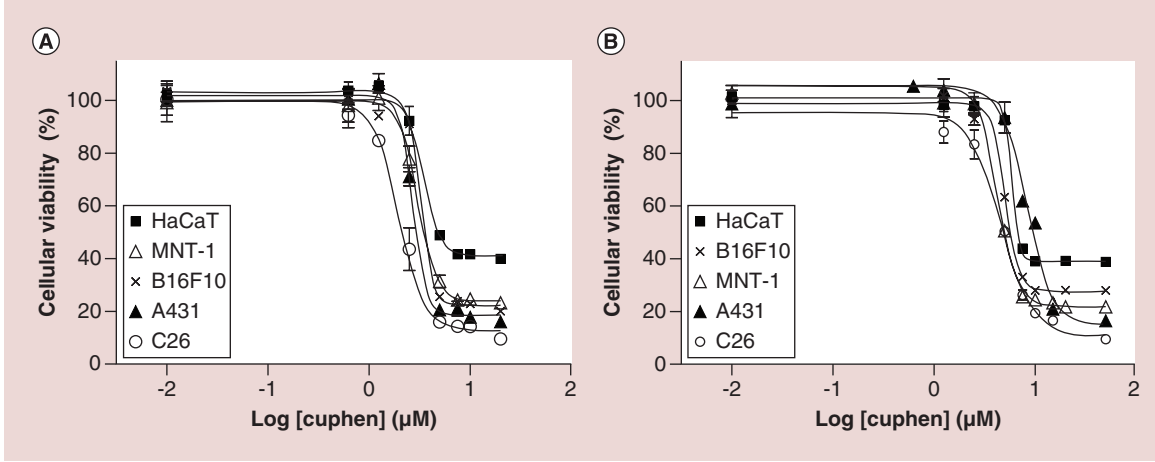

Figure 3. Concentration-dependent inhibition of different cell lines, 72 h after incubation with Cuphen (A) in the free form or (B) incorporated in liposomes (Formulation 2) (MTS assay). Cuphen concentrations ranged from 0.625 to 20 μM. Results are expressed as the mean percentage (%) of control ± SD.

Table 2. Half–inhibitory concentration of Cuphen in the free form or after incorporation into liposomes (F1 and F2), 72 h after incubation in different cell lines.

Cell line	IC_{50} ± SE (μM)		
	Free Cuphen	F1	F2
HaCat	3.4 ± 0.2	5.3 ± 0.2	5.8 ± 0.1
MNT-1	3.1 ± 0.2	4.8 ± 0.2	4.4 ± 0.2
A431	2.7 ± 0.1	10.0 ± 0.8	8.3 ± 0.8
C26	1.8 ± 0.1	5.8 ± 0.4	4.4 ± 0.4
B16F10	3.3 ± 0.3	4.5 ± 0.2	5.1 ± 0.1

Lipid compositions: Formulation 1: ePC; Formulation 2: ePC:Chol:PEG.
IC_{50}: Half-inhibitory concentration.

cancer tissues where AQP3 overexpression has already been demonstrated: two human skin cancer cell lines, the A431 (epidermal carcinoma) [18,47] and the MNT-1 (melanotic neuroectodermal tumor) [48], as well as a nontumorigenic human keratinocyte cell line, HaCaT [49,50]. Aiming for possible future *in vivo* studies, two murine cell lines were also included: B16F10 (for melanoma) [3] and C26 (for colon cancer) [51,52].

In this study, Cuphen proved to be effective by inhibiting cellular viability for all the evaluated cell lines in the range of the concentrations tested (from 0.625 to 20 μM). All dose–response curves presented a similar profile with a maximum decrease on cellular viability of around 80%, except for the healthy cell line HaCaT showing only a 60% maximal reduction in viability (Figure 3A). The IC_{50} values determined are at the low micromolar range (<4 μM) (Table 2).

Cuphen proved to be highly cytotoxic against all the considered tumor cell lines with IC_{50} values around 3 μM. It is worth noting that Cuphen was also cytotoxic against human healthy keratinocytes HaCaT, which was not surprising, since HaCaT cells also express AQP3 [50] although with lower levels than tumor cells. Thus, following these promising results and pursuing the transposition for *in vivo* studies, liposomes containing Cuphen were developed and characterized.

Characterization of Cuphen liposomes

Cuphen was incorporated in liposomes using the dehydration–rehydration method followed by an extrusion step to reduce the mean size of the so-formed nanoformulations [36,53,54]. Due to its hydrophilic properties, Cuphen is only added after formation of the lipid film. Following the lyophilization of the so-formed liposomal suspension, rehydration was performed with HEPES buffer in two steps, in order to increase Cuphen incorporation [37].

Cuphen liposomes were prepared with different lipid compositions, in other words, phospholipids of different phase transition temperatures (Tc), aiming to select the ones that were able to accommodate higher amounts of Cuphen per mol of lipid. Specifically, three neutral phospholipids – ePC (natural phospholipid), DMPC and DPPC (synthetic phospholipids) were used as major constituents of Cuphen nanoformulations that present increasing Tc of -6, +23 and +41°C, respectively.

The first series of nanoformulations was based on the natural phospholipid ePC. Considering its high fluidity, Chol was included in the lipid composition to decrease the membrane permeability and consequently to enhance the stability of the incorporated Cuphen. The inclusion of positively charged surfactant, SA, was also used and the influence of its presence in Cuphen incorporation parameters was compared with neutral nanoformulations. In addition, as it is our purpose to develop long circulating liposomes, another set of lipid compositions containing distearoylphosphatidyl ethanolamine (PEG) was also prepared. In Table 3, the results obtained for different ePC Cuphen formulations are summarized.

All ePC-Cuphen liposomes presented mean sizes ranging from 0.15 to 0.20 μm with polydispersion index below 0.15, evidencing the high homogeneity of all nanoformulations. In terms of loading capacity, ePC formulations without Chol (F1 and F4) were able to accommodate high amounts of Cuphen per μmol of lipid with I.E. values of 54–61%, respectively. The inclusion of Chol in the lipid composition led to a decrease on incorporation parameters (F2 and F3). When included in the lipid composition of liposomes, Chol is inserted within bilayers, influencing membrane fluidity and competing with the accommodation of hydrophobic molecules. These findings are in accordance with the literature [56,57] suggesting that despite its hydrophilic character, Cuphen, may also interact with the lipid bilayers, probably through the ligand phenanthroline, thus explaining the observed I.E. values. Interestingly, in the absence of PEG, all formulations showed similar zeta potential values (-9 ± 1 mV).

Table 3. Physicochemical characterization of Cuphen liposomes.

Formulation	Lipid composition (molar ratio)	Cuphen liposomes				Unloaded liposomes	
		(Cuphen/Lip)f (nmol/μmol)	I.E. (%)	ø (μm) (PI)	Zeta Pot. (mV)	ø (μm) (PI)	Zeta Pot. (mV)
1	ePC	21 ± 7	54 ± 1	0.15 (<0.15)	-9 ± 1	0.14 (0.1)	-4 ± 1
2	ePC:Chol:PEG (1.85:1:0.15)	15 ± 3	47 ± 5	0.16 (<0.15)	-4 ± 1	0.14 (0.1)	-3 ± 1
3	ePC:Chol (2:1)	17 ± 2	44 ± 2	0.20 (<0.10)	-9 ± 1	ND	ND
4	ePC:SA (9.5:0.5)	22 ± 5	61 ± 1	0.16 (<0.15)	-9 ± 1	ND	ND

Results are expressed as mean ± SD.
Initial lipid concentration [Lip]i: 20 μmol/ml; Initial Cuphen [Cuphen]i: 500 nmol/ml; ePC transition temperature: -6°C [55].
I.E. (%): Incorporation efficiency: [(Cuphen/Lip)f]/[(Cuphen/Lip)i] × 100; ø: Mean size of liposomes; PI: Polydispersity index; Zeta Pot.: Zeta potential.

Even for F4, the inclusion of SA was not able to reduce the negative charge of liposomes. In order to clarify these observations, unloaded liposomes with the same lipid composition of F1 and F2 were prepared (Table 3). The results show no differences on the zeta potential values for ePC:Chol:PEG (-3 ± 1 mV), while the ePC-unloaded liposomes displayed less negative values (-4 ± 1 mV) than those observed in the presence of Cuphen (-9 ± 1 mV). The different physicochemical properties observed for F1 point out that Cuphen influences the superficial charge of the vesicles. In addition, nanoformulations of ePC:SA and DMPC:SA with the same molar ratio incorporating Cuphen were prepared and compared regarding the superficial charge and I.E. values. The higher I.E., observed for ePC:SA, resulted in higher negative values for the zeta potential whereas for DMPC:SA the value was positive (+7 ± 1 mV) (data not shown). This finding reinforces the hypothesis of a possible insertion of Cuphen within the liposome membrane, even partial, justifying the changes of the superficial charge when Cuphen is incorporated in ePC (F1).

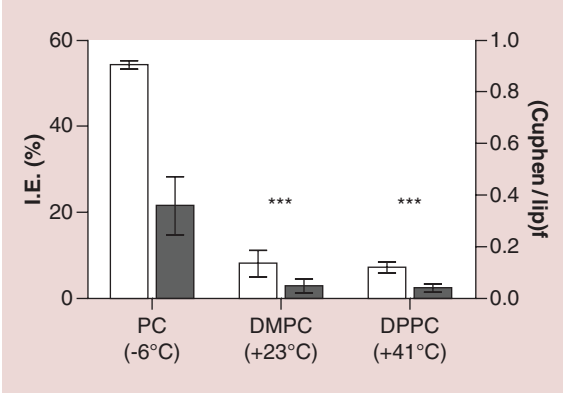

Figure 4. Influence of the bilayer rigidity on Cuphen incorporation parameters: I.E. (%) (white columns) and [Cuphen/Lip]f (gray columns). Comparison between ePC, DMPC and DPPC. Results are expressed as mean ± S.D. ***p < 0.001.

The second set of nanoformulations was designed to evaluate the influence of the rigidity of the main neutral phospholipid on Cuphen incorporation parameters. For this purpose Cuphen liposomes were prepared with ePC, DMPC or DPPC, in other words, phospholipids with increased Tc. As shown in Figure 4, the increase in Tc of the phospholipid used, as major constituent of the lipid bilayer, led to a systematic decrease of Cuphen I.E. from 54 to 6%. F1 and F2 were selected for pursuing *in vitro* tests, as they presented high incorporation parameters and long circulating properties, respectively. Although similar incorporation parameters were achieved for formulation F4, it was not chosen for *in vitro* studies due to SA toxicity reported in the literature [58,59].

Cytotoxicity of Cuphen liposomes

The ability of Cuphen-loaded liposomes to induce cell death was evaluated by the MTS assay for all the cell lines and by the ViaCount assay for one selected cell line to validate results.

MTS assay

The incubation conditions for Cuphen liposomes were established in comparison to the cellular viability at different time-points and by using Cuphen in the free form as a positive control (data not shown). In Figure 3B, the dose–response curves of cells incubated for 72 h with PEG liposomes (F2) and different concentrations of Cuphen (ranging from 0.625 to 20 μM) are shown; while the calculated IC_{50} values

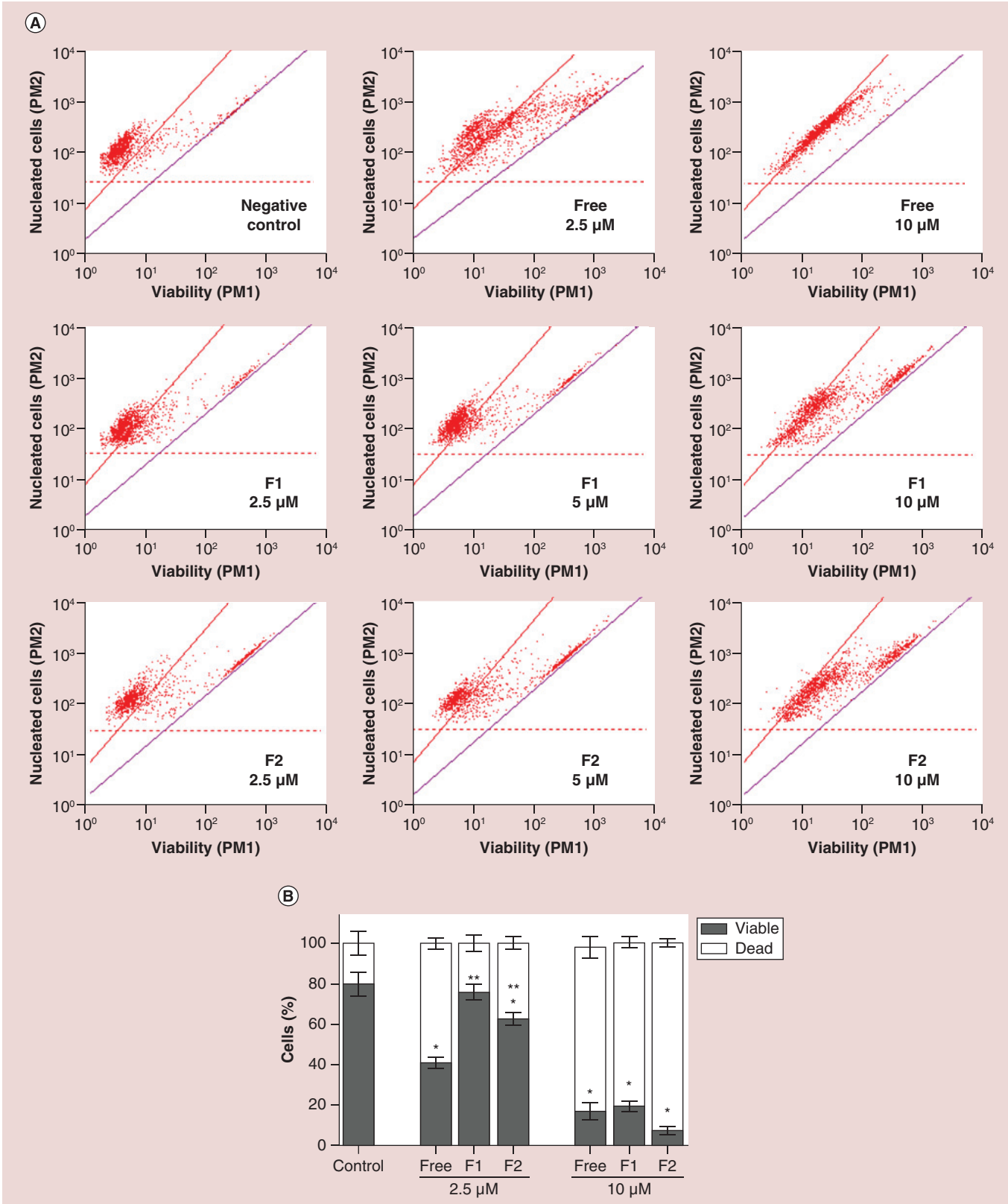

Figure 5. Evaluation of Cuphen formulations effect on cell viability by ViaCount. **(A)** Cell populations obtained by Guava ViaCount flow cytometry after 72 h incubation of MNT-1 melanoma cells with different Cuphen concentrations. **(B)** Percentage of viable and dead cells. Results are expressed as mean ± SEM of at least three different experiments. Lipid compositions: Formulation 1 – ePC; Formulations 2 – ePC:Chol:PEG.
*p < 0.001 from control; **p < 0.001 from the respective free form concentration.

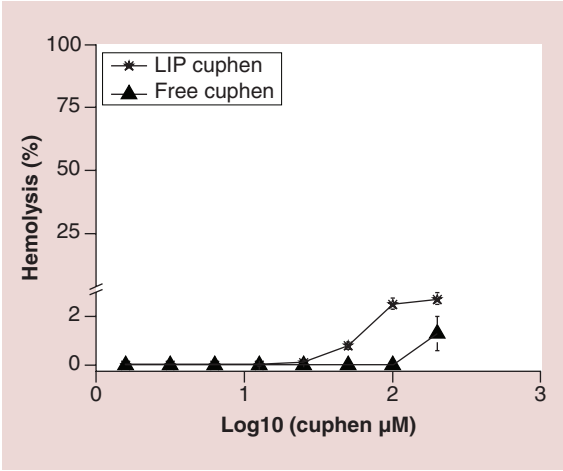

Figure 6. Hemolytic activity of Cuphen formulations in human RBC. Tested Cuphen concentrations ranged from 1 to 200 μM. Lipid composition ePC:Chol:PEG.

are reported in Table 2. The similar cellular viability behavior is reflected by the close cytotoxic profiles.

In general, when incorporated in liposomes, Cuphen was able to exert its cytotoxic effect with IC_{50} values in the low micromolar range (≤10 μM), but slightly higher than the compound alone (see Table 2). The slight increase in the observed IC_{50} values suggests that Cuphen remains incorporated in liposomes and, as such, is not immediately available to exert cytotoxic effects. Similar observations have been reported for other antitumor drugs [60]. As previously observed for the free form of the metallodrug, the efficacy of Cuphen liposomes must not only consider the IC_{50} value (Table 2) but also the survival rates, reflecting the extension of the cytotoxic effect. Our results allowed identifying the maintenance of survival rates, despite the liposomal formulation. In other words, one can represent the cell lines according to the survival rates in the following order: HaCaT>B16F10>MNT-1>A431>C26 with survival rates ranging from 10 to 40% for C26 and HaCaT cells, respectively. The highest cellular survival rate was obtained for the healthy skin cell line, following by the skin cancer cell lines. Accordingly, as the most sensitive cell line, one can identify the murine colon cancer cells

Guava ViaCount assay

The analysis on cell viability obtained by Guava ViaCount flow cytometry corroborates the cytotoxic behavior of the different Cuphen formulations observed with the MTS assay. After 72 h of incubation, both F1 and F2 showed similar effects to the free drug, inducing loss of cell viability by 80% (Figure 5). Thus, the Cuphen-liposome formulation maintained the potent apoptotic properties of the copper complex.

Hemolytic activity of Cuphen

The US FDA recommends that for excipients intended for injectable use, an *in vitro* hemolysis study should be performed at the intended concentration for iv. administration to test for hemolytic potential [61]. The *in vitro* hemolysis assay evaluates hemoglobin release in the plasma (as an indicator of red blood cell lysis) following exposure to a test agent [41]. In the present work, the hemolytic activity against RBCs was used as a marker of a general membrane toxicity effect of Cuphen. In this assay, the percentage of hemolysis of Cuphen in free form or incorporated in liposomes was tested for concentrations up to 200 μM. As shown in Figure 6, the hemolysis was always below 4% ensuring the safety of these formulations for iv. administration. Moreover, the hemolysis caused by the liposome formulation was substantially reduced with respect to the free Cuphen.

In vivo toxicity of Cuphen

Since copper-based compounds may present side effects *in vivo*, before testing the therapeutic effect of Cuphen liposomes, some preliminary toxicity studies were performed. From the best of our knowledge, this is the first report dealing with *in vivo* administration of Cuphen liposomes. Thus, based on the above-mentioned studies showing that Cuphen do not elicit hemolytic activity up to 200 μM, two groups of mice received iv. injections of the metallodrug in the free form or incorporated in liposomes, for 1 week. All mice groups showed normal behavior and body weight as monitored on a daily basis. At the end of injections schedule, mice were sacrificed and organs of interest collected and weighted. Table 4 shows the tissue index

Table 4. Tissue indexes of mice after iv. administration of Cuphen in free and liposomal forms.

Group of mice	Tissue index				
	Liver	Spleen	Kidney	Lung	Heart
Naive	22.8 ± 0.7	6.1 ± 0.3	13.2 ± 0.3	8.4 ± 0.4	7.0 ± 0.4
Free Cuphen	22.0 ± 0.7	5.7 ± 0.2	13.1 ± 0.4	7.6 ± 0.2	7.3 ± 0.1
LIP Cuphen	22.1 ± 0.4	6.7 ± 0.6	13.5 ± 0.5	7.7 ± 0.3	7.0 ± 0.1

Mice received formulations at a dose of 1.5 mg/kg of body weight, three-times a week during 1 week.
Lipid composition ePC:Chol:PEG. Results are expressed as the mean ± SD of five mice per group.

Table 5. Aspartate transaminase and alanine transaminase ratio from plasma samples of mice after iv. administration of Cuphen in free and liposomal forms.

Group of mice	AST/ALT ratio
Naive	0.6 ± 0.1
Free Cuphen	0.8 ± 0.2
LIP Cuphen	0.8 ± 0.2

LIP Cuphen: lipid composition ePC:Chol:PEG. Results are expressed as the mean ± SD of five mice per group.
ALT: Alanine transaminase; AST: Aspartate transaminase.

for the analyzed organs, namely the liver, spleen, kidney, heart and lung of mice receiving Cuphen formulation F2 in comparison with animals receiving free Cuphen and with naive mice, in other words, animals that did not receive any formulation. Tissue index allows the evaluation of changes in a specific organ that may occur related to the whole body weight. Organ weight changes have long been accepted as a sensitive indicator of chemically induced changes to organs [62]. In particular, liver, kidneys and spleen are considered the most valuable in different areas such as pharmaceutical, veterinary, chemical and food/nutritional/consumer [62]. Results showed no statistical significant differences for the tissue indexes among the three groups of mice. The aspartate transaminase (AST) and the alanine transaminase (ALT) blood levels and ratio were also determined, since these are two of the most reliable markers of hepatocellular injury or necrosis [63]. The results are shown in Table 5, proving that no toxic hepatic effects were observed following iv. injection of the Cuphen formulations.

It is also worth mentioning that in general, drug delivery systems in particular liposomes have highly contributed in cancer treatment [29,30]. Liposomal formulations, specifically those containing PEG in the lipid composition, present several advantages over the administration of a free molecule. The iv. administration of PEG liposomes results in a prolonged blood circulation, reduced interaction with plasma proteins and thus allowing a preferential accumulation in tumor tissues. Due to the leaky nature of the tumor-associated blood vessels, liposomes translocate across the capillary endothelium and enter in the interstitial space. The size of the gaps between the endothelial cells lining the tumor capillaries ranges from 100 to 780 nm depending on the cancer type, as opposed to that in a typical normal endothelium of 5–10 nm [64]. In addition, solid tumors are characterized by an inadequate lymphatic drainage. Therefore, there is limited circulatory recovery of the extravasated molecules, resulting in the accumulation of liposomal formulations in the tumor microenvironment. Unlike liposomes, low-molecular-weight drugs are not retained in the tumor site for a longer period of time, since they re-enter the circulation primarily via diffusion [65]. It is clear the importance of using liposomal formulations of Cuphen in particular those containing PEG that present long blood circulation times and consequently high possibility to extravasate to solid tumors.

Conclusion

In the present work, the Cu(II) complex Cuphen, an AQP3 inhibitor, showed potent cytotoxic effects toward different tumor cell lines. Various reports indicate that the high reactivity of Cu(II) ions *in vitro* may have a high damage potential. Therefore, Cuphen was efficiently incorporated in long circulating liposomes and kept the properties resulting from being a potent apoptotic inducer.

The lack of *in vivo* toxicity after parenteral administration renders this nanoformulation attractive to be used against melanoma and colon cancer taking into account the preferential extravasation and accumulation of PEG liposomes in solid tumors. The same strategy can be easily adapted for other metallodrugs such as the gold(III) complex Auphen that shows even higher AQP3 inhibition ability [14]. Further studies are certainly necessary to validate inhibition of AQP3 as the main target responsible for the anticancer effects of Cuphen and similar metal compounds, as well as to assess Cuphen anticancer activity *in vivo*. As a matter of fact, various biomolecules other than AQPs, and different cellular pathways were reported to be likely targets for different families of cytotoxic copper(II) complexes [22]. At this stage of our investigation, we believe that inhibition of AQPs may still occur extracellularly upon release of Cuphen from the liposome formulation, being the main function of the formulation not necessarily to increase the uptake of Cuphen in cancer cells, but to enhance its stability as well as its accumulation in cancerous tissues via the enhanced permeability and retention (EPR) effect.

Future perspective

Clinical and preclinical studies evidence the overexpression of AQPs in a high number of cancers. In this

sense, the development of selective AQP inhibitors represents a new therapeutic strategy in cancer treatment. Metallodrugs of copper and gold (as those included in the present work) are potential candidates to fulfill this need. However, to promote a preferential targeting to tumor sites, the design and development of novel technologies based on nanostructured materials, such as liposomes, acting mainly as vectors for metallodrug delivery or simply as protectors of the complexes for amplifying their activities and reducing their degradation, constitutes a stimulating research area. We expect that these strategies can raise the interest of the pharmaceutical companies allowing the introduction of novel metallodrugs nanoformulations in the market as anticancer chemotherapeutics.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.futuremedicine.com/doi/full/10.2217/nnm-2016-0086

Financial & competing interests disclosure

The authors acknowledge EU COST action CM1106 for funding and fruitful discussion with the participants of the networks. The work was partially funded by iMed.ULisboa (UID/DTP/04138/2013) from Fundação para a Ciência e a Tecnologia (FCT), Portugal. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Executive summary

- Cuphen is a potent copper-based aquaporin inhibitor with cytotoxic effect against melanoma and colon cancer cell lines, with IC_{50} values in the micromolar range.
- Cuphen was successfully incorporated into long circulating liposomes following a suitable selection of lipid compositions.
- The cytotoxic properties of Cuphen after incorporation in liposomes were preserved.
- Cuphen liposomes exhibit appropriate characteristics for *in vivo* administration, thus allowing the *in vivo* targeting and accumulation in tumor sites.
- Cuphen liposomes did not elicit hemolytic activity.
- Cuphen liposomes did not display toxicity after *in vivo* administration.
- Cuphen-PEG liposomes may be considered a very attractive nanoformulation with therapeutic potential against melanoma and colon cancer due to their preferential extravasation and accumulation in solid tumors.

References

Papers of special note have been highlighted as: • of interest; •• of considerable interest

1 Soveral G, Nielsen S, Casini A. *Aquaporins in Health and Disease: New Molecular Targets for Drug Discovery*. CRC Press, Taylor & Francis Group, FL, USA (2016).

•• **This book examines the aquaporin physiological role in health and disease, describes chemical inhibition by small-molecule compounds that may present drug development opportunities and explores future challenges and possibilities in aquaporin pharmacology.**

2 Agre P, Sasaki S, Chrispeels M. Aquaporins: a family of water channel proteins. *Am. J. Physiol.* 265(3 Pt 2), F461 (1993).

3 Verkman AS, Hara-Chikuma M, Papadopoulos MC. Aquaporins – new players in cancer biology. *J. Mol. Med.* 86(5), 523–529 (2008).

4 Papadopoulos MC, Saadoun S. Key roles of aquaporins in tumor biology. *Biochim. Biophy. Acta* 1848(10 Pt B), 2576–2583 (2015).

5 Direito I, Madeira A, Brito MA, Soveral G. Aquaporin-5: from structure to function and dysfunction in cancer. *Cell. Mol. Life Sci.* 73(8), 1623–1640 (2016).

6 Sougrat R, Morand M, Gondran C *et al.* Functional expression of AQP3 in human skin epidermis and reconstructed epidermis. *J. Invest. Dermatol.* 118(4), 678–685 (2002).

7 Wang J, Tanji N, Kikugawa T, Shudou M, Song X, Yokoyama M. Expression of aquaporin 3 in the human prostate. *Int. J. Urol.* 14(12), 1088–1092; discussion 1092 (2007).

8 Kusayama M, Wada K, Nagata M *et al.* Critical role of aquaporin 3 on growth of human esophageal and oral squamous cell carcinoma. *Cancer Sci.* 102(6), 1128–1136 (2011).

9 Hara-Chikuma M, Verkman AS. Prevention of skin tumorigenesis and impairment of epidermal cell proliferation by targeted aquaporin-3 gene disruption. *Mol. Cell. Biol.* 28(1), 326–332 (2008).

10 Hara-Chikuma M, Verkman AS. Aquaporin-3 facilitates epidermal cell migration and proliferation

during wound healing. *J. Mol. Med. (Berl.)* 86(2), 221–231 (2008).

11 Verkman AS, Anderson MO, Papadopoulos MC. Aquaporins: important but elusive drug targets. *Nat. Rev. Drug Discov.* 13(4), 259–277 (2014).

12 De Almeida A, Soveral G, Casini A. Gold compounds as aquaporin inhibitors: new opportunities for therapy and imaging. *Med. Chem. Comm.* 5(10), 1444–1453 (2014).

•• **An overview of the importance of aquaporins in various diseases and a detailed description of the state-of-the-art progresses in the discovery of new inhibitors and a description of their possible application.**

13 Martins AP, Marrone A, Ciancetta A *et al.* Targeting aquaporin function: potent inhibition of aquaglyceroporin-3 by a gold-based compound. *PLoS ONE* 7(5), 1–14 (2012).

14 Martins AP, Ciancetta A, De Almeida A *et al.* Aquaporin inhibition by gold(III) compounds: new insights. *Chem. Med. Chem.* 8(7), 1086–1092 (2013).

15 Messori L, Abbate F, Marcon G *et al.* Gold(III) complexes as potential antitumor agents: solution chemistry and cytotoxic properties of some selected gold(III) compounds. *J. Med. Chem.* 43(19), 3541–3548 (2000).

16 Casini A, Kelter G, Gabbiani C *et al.* Chemistry, antiproliferative properties, tumor selectivity, and molecular mechanisms of novel gold(III) compounds for cancer treatment: a systematic study. *J. Biol. Inorg. Chem.* 14(7), 1139–1149 (2009).

17 Serratrice M, Edafe F, Mendes F *et al.* Cytotoxic gold compounds: synthesis, biological characterization and investigation of their inhibition properties of the zinc finger protein PARP-1. *Dalton Trans.* 41(11), 3287–3293 (2012).

18 Serna A, Galan-Cobo A, Rodrigues C *et al.* Functional inhibition of aquaporin-3 with a gold-based compound induces blockage of cell proliferation. *J. Cell. Physiol.* 229 1787–1801 (2014).

• **Describes the inhibitory effect of a metallodrug on proliferation of cells expressing AQP3 suggesting a targeted therapeutic effect on carcinomas with high AQP3 expression.**

19 Bertrand B, Casini A. A golden future in medicinal inorganic chemistry: the promise of anticancer gold organometallic compounds. *Dalton Trans.* 43(11), 4209–4219 (2014).

• **Provides an overview of the main families of organometallic gold compounds as anticancer agents.**

20 De Vizcaya-Ruiz A, Rivero-Muller A, Ruiz-Ramirez L *et al.* Induction of apoptosis by a novel copper-based anticancer compound, casiopeina II, in L1210 murine leukaemia and CH1 human ovarian carcinoma cells, *Toxicol. In Vitro* 14(1), 1–5 (2000).

21 Trejo-Solis C, Palencia G, Zuniga S *et al.* Cas IIgly induces apoptosis in glioma C6 cells *in vitro* and *in vivo* through caspase-dependent and caspase-independent mechanisms. *Neoplasia* 7(6), 563–574 (2005).

22 Santini C, Pellei M, Gandin V, Porchia M, Tisato F, Marzano C. Advances in copper complexes as anticancer agents. *Chem. Rev.* 114(1), 815–862 (2014).

23 Hu C, Li X, Wang W, Zhang R, Deng L. Metal-N-heterocyclic carbene complexes as anti-tumor agents. *Curr. Med. Chem.* 21(10), 1220–1230 (2014).

24 Vértiz G, García-Ortuño LE, Bernal JP *et al.* Pharmacokinetics and hematotoxicity of a novel copper-based anticancer agent: casiopeina III-Ea, after a single intravenous dose in rats. *Fundam. Clin. Pharmacol.* 28(1), 78–87 (2014).

25 Wani WA, Prashar A, Shreaz A, Gómes-Ruiz S. Nanostructured materials functionalized with metal complexers: in search of alternatives for administering anticancer metallodrugs. *Coord. Chem. Rev.* 312, 67–98 (2016).

26 Lopez T, Ortiz-Islas E, Guevara P, Gómez E. Catalytic nanomedicine technology: copper complexes loaded on titania nanomaterials as cytotoxic agents of cancer cell. *Int. J. Nanomed.* 8, 581–592 (2013).

27 El Brahmi N, El Kazzouli S, Mignani SM *et al.* Original multivalent copper(II)-conjugated phosphorus dendrimers and corresponding mononuclear copper(II) complexes with antitumoral activities. *Mol. Pharm.* 10(49, 1459–1464 (2013).

28 Allen TM, Cullis PR. Drug delivery systems: entering the mainstream. *Science* 303(5665), 1818–1822 (2004).

• **Advantages of drug delivery systems for *in vivo* delivery of new drugs.**

29 Torchilin VP. Recent advances with liposomes as pharmaceutical carriers. *Nat. Rev. Drug Discov.* 4(2), 145–160 (2005).

30 Torchilin VP. Targeted pharmaceutical nanocarriers for cancer therapy and imaging. *AAPS J.* 9(2), E128–E147 (2007).

31 Tila D, Ghasemi S, Yazdani-Arazi SN, Ghanbarzadeh S. Functional liposomes in the cancer-targeted drug delivery. *J. Biomater Appl.* 30(1), 3–16 (2015).

32 Maeda H, Sawa T, Konno T. Mechanism of tumor-targeted delivery of macromolecular drugs, including the EPR effect in solid tumor and clinical overview of the prototype polymeric drug SMANCS. *J. Control. Release* 74(1–3), 47–61 (2001).

• **The mechanism of the enhanced permeability and retention effect and the advantages for cancer treatment.**

33 Maeda H, Wu J, Sawa T, Matsumura Y, Hori K. Tumor vascular permeability and the EPR effect in macromolecular therapeutics: a review. *J. Control. Release* 65(1–2), 271–284 (2000).

34 Casini A, Diawara MC, Scopelliti R, Zakeeruddin SM, Gratzel M, Dyson PJ. Synthesis, characterisation and biological properties of gold(III) compounds with modified bipyridine and bipyridylamine ligands. *Dalton Trans.* 39(9), 2239–2245 (2010).

35 Block BP, Bailar JC. The reaction of gold(III) with some bidentate coördinating groups. *J. Am. Chem. Soc.* 73(10), 4722–4725 (1951).

36 Cruz MEM, Gaspar MM, Lopes F, Jorge JS, Perez-Soler R. Liposomal L-asparaginase: *in vitro* evaluation. *Int. J. Pharm.* 96(1–3), 67–77 (1993).

37 Lasch J, Weissig V, Brandl M. *Preparation of Liposomes*. Oxford University Press Inc., NY, USA (2003).

38 Rouser G, Fkeischer S, Yamamoto A. Two dimensional thin layer chromatographic separation of polar lipids and determination of phospholipids by phosphorus analysis of spots. *Lipids* 5(5), 494–496 (1970).

39 Cory AH, Owen TC, Barltrop JA, Cory JG. Use of an aqueous soluble tetrazolium/formazan assay for cell growth assays in culture. *Cancer Commun.* 3(7), 207–212 (1991).

40 Mansoor TA, Borralho PM, Luo X, Mulhovo S, Rodrigues CM, Ferreira MJ. Apoptosis inducing activity of benzophenanthridine-type alkaloids and 2-arylbenzofuran neolignans in HCT116 colon carcinoma cells. *Phytomedicine* 20(10), 923–929 (2013).

• **Describe the *in vitro* tests for evaluating the cellular antiproliferative effect of promising molecules.**

41 Gaspar MM, Calado S, Pereira J *et al.* Targeted delivery of paromomycin in murine infectious disease s through association to nano lipid systems. *Nanomedicine* 11(7), 1851–1860 (2015).

•• **Potential clinical significance for the treatment of infectious diseases using liposomes.**

42 Burkitt MJ, Milne L, Nicotera P, Orrenius S. 1,10-Phenanthroline stimulates internucleosomal DNA fragmentation in isolated rat-liver nuclei by promoting the redox activity of endogenous copper ions. *Biochem. J.* 313(Pt 1), 163–169 (1996).

43 Bales BC, Kodama T, Weledji YN, Pitie M, Meunier B, Greenberg MM. Mechanistic studies on DNA damage by minor groove binding copper–phenanthroline conjugates. *Nucleic Acids Res.* 33(16), 5371–5379 (2005).

44 Sigman DS, Graham DR, D'aurora V, Stern AM. Oxygen-dependent cleavage of DNA by the 1,10-phenanthroline. Cuprous complex. Inhibition of *Escherichia coli* DNA polymerase I. *J. Biol. Chem.* 254(24), 12269–12272 (1979).

45 Sigman DS, Landgraf R, Perrin DM, Pearson L. Nucleic acid chemistry of the cuprous complexes of 1,10-phenanthroline and derivatives. *Met. Ions Biol. Syst.* 33, 485–513 (1996).

46 Zelenina M, Tritto S, Bondar AA, Zelenin S, Aperia A. Copper inhibits the water and glycerol permeability of aquaporin-3. *J. Biol. Chem.* 279(50), 51939–51943 (2004).

47 Hara-Chikuma M, Watanabe S, Satooka H. Involvement of aquaporin-3 in epidermal growth factor receptor signaling via HO transport in cancer cells. *Biochem. Biophys. Res. Commun.* 471(4), 603–609 (2016).

48 Bin BH, Bhin J, Yang SH *et al.* Hyperosmotic stress reduces melanin production by altering melanosome formation. *PLoS ONE* 9(8), e105965 (2014).

49 Cao C, Wan S, Jiang Q *et al.* All-trans retinoic acid attenuates ultraviolet radiation-induced down-regulation of aquaporin-3 and water permeability in human keratinocytes. *J. Cell. Physiol.* 215(2), 506–516 (2008).

50 Ji C, Yang Y, Yang B *et al.* Trans-Zeatin attenuates ultraviolet induced down-regulation of aquaporin-3 in cultured human skin keratinocytes. *Int. J. Mol. Med.* 26(2), 257–263 (2010).

51 Li A, Lu D, Zhang Y *et al.* Critical role of aquaporin-3 in epidermal growth factor-induced migration of colorectal carcinoma cells and its clinical significance. *Oncol. Rep.* 29(2), 535–540 (2013).

52 Moon C, Soria JC, Jang SJ *et al.* Involvement of aquaporins in colorectal carcinogenesis. *Oncogene* 22(43), 6699–6703 (2003).

53 Gaspar MM, Perez-Soler R, Cruz ME. Biological characterization of L-asparaginase liposomal formulations. *Cancer Chemoth. Pharm.* 38(4), 373–377 (1996).

•• **Describes the use of phospholipids such as phosphatidylinositol (PI) and monosialoganglioside (GM1) as the constituents on the lipid composition of the first generation of long circulating liposomes.**

54 Gaspar MM, Cruz A, Penha AF *et al.* Rifabutin encapsulated in liposomes exhibits increased therapeutic activity in a model of disseminated tuberculosis. *Int. J. Antimicrobial Agents* 31(1), 37–45 (2008).

55 Gunstone FD, Harwood JL, Padley FB. In: *The Lipid Handbook.* Chapman Hall (1986).

56 Blume G, Cevc G. Molecular mechanism of the lipid vesicle longevity *in vivo*. *Biochim. Biophys. Acta* 1146(2), 157–168 (1993).

57 Constantino L, Cruz MEM, Mehta R, Lopez-Berestein G. Formulation and toxicity of liposomes containing Rifampicin. *J. Liposome Res.* 3(2), 275–301 (1993).

58 Takano S, Aramaki Y, Tsuchiya S. Physicochemical properties of liposomes affecting apoptosis induced by cationic liposomes in macrophages. *Pharm. Res.* 20(7), 962–968 (2003).

59 Epstein-Barash H, Gutman D, Markovsky E *et al.* Physicochemical parameters affecting liposomal bisphosphonates bioactivity for restenosis therapy: internalization, cell inhibition, activation of cytokines and complement, and mechanism of cell death. *J. Control. Release* 146(2), 182–195 (2010).

60 Fens MH, Hill KJ, Issa J *et al.* Liposomal encapsulation enhances the antitumour efficacy of the vascular disrupting agent ZD6126 in murine B16.F10 melanoma. *Br. J. Cancer* 99(8), 1256–1264 (2008).

61 US FDA. Guidance for Industry – nonclinical studies for the safety evaluation of pharmaceutical excipients. 1–9 (2005).

62 Michael B, Yano B, Sellers RS *et al.* Evaluation of organ weights for rodent and non-rodent toxicity studies: a review of regulatory guidelines and a survey of current practices. *Toxicol. Pathol.* 35(5), 742–750 (2007).

63 Giboney PT. Mildly elevated liver transaminase levels in the asymptomatic patient. *Am. Fam. Physician* 71(6), 1105–1110 (2005).

64 Haley B, Frenkel E. Nanoparticles for drug delivery in cancer treatment. *Urol. Oncol.* 26(1), 57–64 (2008).

65 Deshpande PP, Biswas S, Torchilin VP. Current trends in the use of liposomes for tumor targeting. *Nanomedicine* 8(9), 1509–1528 (2013).